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Implementing a bioassay to screen molecules for antiepileptogenic activity: chronic pilocarpine versus subdudral haematoma models

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***Background*:** There is a need to discover novel chemical compounds that will inhibit the pathological process of epileptogenesis (i.e. agents that will prevent the long-term formation of an active seizure focus following a brain insult). The goal of this paper is to identify a bioassay of value in drug design when screening new chemical entities as putative antiepileptogenic agents.
***Methods*:** We focused on two models: the pilocarpine chronic seizure model of spontaneous recurrent seizures (SRSs) and a chronic subdural haematoma model of SRSs. Both models were evaluated using more than 20 Sprague–Dawley rats for each model.
***Results*:** In the pilocarpine-induced model of SRSs, 80% of animals went on to develop SRSs when the dose of pilocarpine was 380 mg/kg i.p. In 50 animals that developed SRSs, the average number of seizures per 15 days of observation was 3.8 seizures with a range of 2–23 seizures per 15-day period. The chronic subdural model was inefficient in producing SRSs.
***Conclusions*:** A pilocarpine-induced SRS model of epilepsy affords a reliable model of epileptogenesis suitable for evaluating new chemical entities as putative antiepileptogenics.

Key words: epilepsy; pilocarpine; haematoma; epileptogenesis; animal models.

INTRODUCTION

There is a need to discover novel chemical compounds that will inhibit the pathological process of epileptogenesis (i.e. agents that will prevent the long-term formation of an active seizure focus following a brain insult). Epileptogenesis is a slow, insidious biochemical and morphological process whereby normal brain is transformed (after a 'latency period') into pathologically excitable neural tissue that is susceptible to spontaneous recurrent seizures (SRSs)[1,2]. Most therapies currently available for the treatment of epilepsy do not possess antiepileptogenic properties. Various controlled studies on the efficacy of traditional anticonvulsant drugs as prophylactic agents against epilepsy have failed to show protective effects for agents such as phenytoin or carbamazepine[3,4]. These currently available anticonvulsant medications do not specifically target epileptogenesis, but rather seek to inhibit ictogenesis—the rapidly propagating electrical and chemical event whereby aberrant electrical hyper-excitability spreads from the seizure focus giving rise to the clinical manifestations of a seizure[2,5,6]. The market for anti-ictogenic (anticonvulsant, anti-seizure) agents has been well served over the past century with traditional agents such as phenobarbital, phenytoin and carbamazepine and with newer generation medications such as lamotrigine. It is a neuropharmacologic priority that the design strategy must now change from anti-seizure agents to antiepileptogenic agents.

Central to a comprehensive programme targeting the discovery of antiepileptogenic agents is the need for an appropriate animal model. This model not only must accurately reflect the biology of epileptogenesis, but also must be useful for the facile screening of new chemical entities for potential bioactivity. Current widely used assays, such as the maximal

electroshock (MES) model and the pentylenetetrazol (ScMet) model (and other related chemoconvulsant assays) are models of seizures, rather than models of epileptogenesis. Although kindling has been put forward as a model for epileptogenesis, the applicability of kindling as a procedure for identifying potential therapeutics for epilepsy has been debated[7,8]. Accordingly, there is a continuing need for relevant models of epileptogenesis that can be used in the screening of new chemical entities.

The goal of this paper is to identify a bioassay of potential value in drug design and discovery when screening new chemical entities as putative antiepileptogenic agents. The drug design/medicinal chemistry literature does not currently employ an 'accepted' model of epileptogenesis. Accordingly, we describe our attempts to identify an *in vivo* model of epileptogenesis that reliably produces SRSs in an experimental model that also could permit the screening of test molecules for potential antiepileptogenic activity. After preliminary evaluation of several models, we focused on two models for potential development: the pilocarpine chronic seizure model (adapted from Mello *et al.*[9,10]) and a chronic subdural haematoma model (adapted from Willmore and co-workers[11,12]).

METHODS AND MATERIALS

The pilocarpine chronic seizure model of spontaneous recurrent seizures

As a first step it was crucial that the model be implemented in a manner that would enable accuracy and reproducibility. Initial experiments were performed using groups of 10 rats. On day 1, a group of 10 male Sprague–Dawley rats (175–200 g, Charles River) was given methylscopolamine (1 mg/kg i.p.) to minimise the peripheral effects of pilocarpine. Thirty minutes later, the animals were injected with pilocarpine (380 mg/kg i.p., Aldrich Chemical Co.). Approximately 15 minutes later, 80% of the rats entered a state of convulsive *status epilepticus*. Animals that did not undergo continuous seizure activity were not used any further in the study. Initially, the animals typically exhibited ear twitching, head jerking and body rearing, followed by several minutes in which this behaviour diminished slightly; then the body movements started again and a constant seizure state persisted for hours. If the animal was able to sustain the continuous seizure activity while remaining on its feet (moderate severity) then the *status epilepticus* was allowed to last for 3 hours. However, if the seizure severity was severe and the animal was lying on its side, then the seizure was terminated after 2 hours. In our experience, animals that underwent a severe seizure for 3 hours did not survive the experiment. The mortality rate during the *status epilepticus* period was 20%. The behavioural aspects of the seizures were stopped at the desired time (2 hours for severe seizures; 3 hours for moderate severity) by the administration of diazepam (4 mg/kg i.p.). This injection stopped the seizure activity after an average of 2.8 minutes. Following the *status epilepticus* episode, the hydration level of the animals was supported by the administration of 20% sucrose Gatorade solution (2.5 ml, p.o.) and Ringer's lactate solution (5 ml i.p.) once a day for 7 days. Additionally, a portion of the rat chow pellets was ground into a paste using water. The animals were weighed each day. A 20% body weight loss was typical following the *status epilepticus*; normal eating patterns with evidence of weight was typically recovered by day 3.

Rats were placed individually in clear Plexiglas cages; food and water were provided *ad libitum*. The light/dark cycle was maintained at 12 hours/12 hours. On day 21, the cages were placed in the videotaping room. The animals were placed in front of video camera stations and were videotaped for 8 hours/day, 5 days/week for 8 weeks. Six cages were placed in each taping station with a contrasting dark background behind the cages. The number of seizures was determined by individuals viewing the videotapes. Quality control and quality assurance methods were put in place to verify the seizure count data. Every tenth videotape produced was double-viewed to check the agreement between the viewers.

Following 2 months of videotaping the animals were euthanised on day 81. In order to prepare for histological analysis of the brain two perfusion solutions were prepared containing: (1) 0.37% sulphide solution (pH 7.2; 11.7 g $Na_2S{\cdot}9H_2O$ plus 11.9 g $NaH_2PO_4{\cdot}H_2O$ made up to 1000 ml; (2) 10% neutral buffered formalin (NBF; 90 ml of 37% formaldehyde, 3.6 g $NaH_2PO_4{\cdot}H_2O$ and 5.85 g $Na_2HPO_4{\cdot}7H_2O$) dissolved in 810 ml of distilled water[13]. The animals were anaesthetised using sodium pentobarbital (65 mg/kg i.p.) and the thoracic cavity was exposed. The descending aorta was tied off with a ligature. A needle containing the sulphide perfusion solution was then placed into the left ventricle and clamped into place. Another needle was rinsed with heparin (10 000 USP units/ml) and used to withdraw 5 ml of blood from the heart. Using scissors a nick was then placed in the right atrium. The 60 ml sulphide perfusion solution was pushed through followed immediately by 60 ml of neutral buffer formalin. The heart, lungs, liver, kidneys and brain were then removed. The brain was placed in a vial and covered with neutral buffer formalin. The other organs were weighed and measured and a gross examination was performed.

As discussed in Section 'RESULTS', 8 of 10 rats developed SRSs. Accordingly, additional refining experiments were performed. (i) We experimented with varying doses of pilocarpine. Higher doses of pilocarpine (400 mg/kg) produced unacceptably high mortality (6/10 mortality); lower doses of pilocarpine (350 mg/kg) produced unacceptably low rates of SRS development (4/10 developed recurrent seizures). (ii) To ensure that the behavioural manifestations of the seizure corresponded with electrographic seizures, a subset of 10 rats had EEG's recorded. All rats with SRSs showed generalised spike and sharply contoured epileptiform discharges during clinically apparent seizure activity manifesting as whisker twitching, tail extension, body twisting, piloerection, and body rearing with pedalling of the forepaws. (iii) To ensure that the behavioural manifestations of this model correspond with discernible pathology, a subset of 10 rats had post-mortem brain histology examinations performed on cortical (frontal, parietal, temporal and occipital), subcortical and brainstem tissue.

In accord with previous studies by Mello and co-workers[10, 13–16], the SRS model produced neuronal loss and damage in the hippocampus and adjacent limbic structures, paralleling temporal lobe epilepsy. To ensure that the SRS model did not produce systemic non-CNS pathology which could be inappropriately ascribed to a candidate chemical compound undergoing screening (thereby limiting its promotion as a drug candidate), all animals that were videotaped had post-mortem examinations of their organ systems. Pathological examination of heart, lungs, kidneys, liver, stomach, and intestines showed no evidence of gross pathology.

The chronic subdural haematoma model of spontaneous recurrent seizures

Male Sprague–Dawley rats were also used for this model. Each rat was anaesthetised with sodium pentobarbital (65 mg/kg i.p.). The underside of one of the rear legs was shaved. A small incision was made to expose the femoral vein and artery. A heparin solution (0.05 ml, 1000 units/ml) was drawn up into a syringe just prior to blood collection. Blood was drawn into the needle (0.3 ml total of heparin solution and blood) by inserting a 25 G needle into the femoral vein. After removing the needle, pressure was applied to the injection site to stop any bleeding. The incision was sutured. Next, the animal was placed in a stereotactic head frame. A rectal thermostat with an attached heat lamp was set up to ensure maintenance of body temperature. A midline incision was made through the scalp and the skull was exposed. The skull plate and meninges were removed over target area of the left temporal lobe. Then, 10 μl of venous blood/heparin (previously drawn from the femoral vein) was slowly injected 1.0 mm into the temporal lobe cortex over 10 minutes. The skull plate was then replaced, the dura was not repaired, and the scalp was closed. The animal was removed from the head frame, and allowed to recover. Two weeks later the animal was placed in a video camera recording station. The rats were videotaped 8 hours/day, 5 days/week for 6 weeks. The number of seizures was counted from the videotapes. On day 56, each rate was anaesthetised (with sodium pentobarbital), and then euthanised by cardiac puncture. A fixative solution was perfused to prepare the brain for histological analysis (as discussed above for chronic pilocarpine model).

The brains were removed and stored in paraffin. The organs were examined for abnormalities. Pathological examination of the brain confirmed that blood had been injected to a depth of 1.0 mm into the cortex with the formation of an adjacent haematoma.

As discussed in Section 'RESULTS', only 1/10 rats developed spontaneous seizures. In an attempt to improve this frequency of epilepsy, a number of modifications were pursued in groups of four rats. Another series of experiments was performed in which a foreign body (blood soaked surgical sponge) was left on the cortex of the brain over the point of injection into the cortex. None of these animals developed seizures. Alternatively, rather than injecting the blood into the cortex, an attempt was made merely to raise a subdural haematoma on the surface of the sensorimotor cortex without insertion of blood into the parenchyma of the brain. None of these animals developed seizures.

RESULTS

In the pilocarpine induced model of SRSs, 80% of animals went on to develop SRSs when the dose of pilocarpine was 380 mg/kg i.p. In 50 animals that developed SRSs, the average number of seizures per 15 days of observation was 3.8 seizures with a range of 2–23 seizures per 15 day period. When the pilocarpine dose was raised to 400 mg/kg, the mortality rate was 80% (8/10); when the pilocarpine dose was lowered to 350 mg/kg, the number of animals developing SRSs fell to 40% (4/10). Pathological examination of the brains of animals that developed SRSs revealed neuronal loss and damage in the hippocampus and related limbic structures, paralleling temporal lobe epilepsy. Electroencephalographic recordings of animals with SRSs revealed epileptiform discharges during clinically apparent seizure activity manifesting as whisker twitching, tail extension, body twisting, piloerection, and body rearing with pedalling of the forepaws.

In the subdural haematoma induced model of SRSs, 10% of animals went on the develop SRSs. This one animal only demonstrated one seizure during the entire observation period. When a foreign body was inserted (blood soaked sponge) 0/4 animals developed seizures. When the haematoma was injected subdurally without blood injection into cortex, 0/4 animals developed seizures.

DISCUSSION

Since Merritt and Putnam's historic discovery of phenytoin in the late 1930s, there have been many models put forth to screen novel chemicals as potential agents for the treatment of epilepsy (e.g. MES, ScMet, strychnine, bicuculline, picrotoxin, allylglycine, isoniazid, 3-mercaptoproprionate, beta-carboline, penicillin, tungstic acid)[17]. Virtually all of these models have been models of seizures (ictogenesis) and not models of chronic epileptogenesis. Although models such as the MES and ScMet assays have been used extensively, they have been useful in finding anticonvulsants, not antiepileptogenics. Since the need for antiepileptogenics is emerging as a neuropharmacologic priority, the need for an appropriate animal model with which to discover antiepileptogenics is also a priority.

Whether kindling is such an animal model is open to debate. As an experimental technique, kindling has been available for many years; however, it has failed to yield an antiepileptogenic drug. Kindling refers to the process whereby a subthreshold stimulus (usually a focal electrical stimulation of a limbic or cortical structure) repeated over days or weeks becomes a convulsant stimulus[18]. Kindling is a relatively time consuming procedure as it requires the chronic implantation of stimulating electrodes in the amygdala, hippocampus, or cortex, and daily electrical stimulation with scoring of either the after-discharge duration or of the motor seizure response. Furthermore, the relationship between kindling and spontaneous human epilepsy remains controversial. Because the kindled seizure is evoked rather than spontaneous and lacks a definable latent period, kindling is not considered to be an ideal model of epileptogenesis[19]. Another criticism of the kindling model as an assay with which to identify a potential antiepileptogenic compound is that the test substance is administered before the kindling stimulation. Finally, even though a drug might be found to retard the kindling process, the fully kindled rat does not normally exhibit spontaneous seizures; therefore, it is impossible to conclude whether the ability of the drug to block kindling will inhibit the fundamental mechanistic processes of epileptogenesis[19]. Whether kindling is an appropriate animal model with which to identify antiepileptogenic compounds remains uncertain; therefore, the search for a possible alternative persists.

In attempting to identify an alternative to the kindling model for drug molecule screening, a number of possibilities were considered. As stated above, most current animal models are models of seizures rather than models of epileptogenesis. In a recent review of animal models of epileptogenesis, White discusses three general classes of models, other than kindling: *status epilepticus* (e.g. pilocarpine-induced), brain injury (e.g. subdural haematoma or $FeCl_2$ induced, fluid percussion, cortical undercut), and age-specific models (e.g. neonatal hypoxia, neonatal hyperthermia)[19]. In addition to these models, other workers have described the onset of seizures following focal application of metal salts, in particular aluminum, in monkey and cat[17]. These various models all exhibit strengths and weaknesses. For instance, the fluid percussion model does not produce spontaneous seizures. Likewise, the neonatal hypoxia and hyperthermia models also do not yield spontaneous seizures. The use of metallic salts may be more relevant to ictogenesis than to epileptogenesis. Accordingly, we elected to evaluate both a *status epilepticus* (pilocarpine-induced) and a brain injury (subdural haematoma) model.

The adaptation of the SRS model of epileptogenesis as presented in this study is a model that strives to represent the human situation in which an antiepileptogenic medication would be given following a head injury. The *status epilepticus* model is analogous to recurrent seizures after prolonged febrile convulsions; the subdural haematoma model represents the pathogenesis of post-traumatic epilepsy. These SRS models thus have biological and clinical relevance.

In the comparison of the *status epilepticus* versus chronic subdural models presented in this study, the *status epilepticus* model was better. The chronic subdural model produced few if any seizures. The *status epilepticus* model produced numerous spontaneous seizures with high reliability after an appropriate latent period. The seizures correlated with EEG abnormalities and with histopathological changes. If a test compound was administered after the *status epilepticus*, this model could be used to evaluate new chemical entities as putative antiepileptogenic agents.

Despite its obvious strengths, the pilocarpine-induced SRS model also has limitations from the perspective of being an assay for screening new chemical entities. Although the SRS model is biologically relevant, the time requirements and the large amount of labour involved in the videotape viewing is a major drawback of this screening program. Nevertheless, this assay does provide a model of epileptogenesis rather than merely a model of seizures, and could be used to evaluate novel compounds. The ultimate suc-

cess of the pilocarpine induced SRS model to identify new antiepileptogenic agents must await its successful application.

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